Kinetic study of the processing by dipeptidyl-peptidase IV/CD26 of neuropeptides involved in pancreatic insulin secretion

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Abstract Dipeptidyl-peptidase IV (DPPIV/CD26) metabolizes neuropeptides regulating insulin secretion. We studied the in vitro steady-state kinetics of DPPIV/CD26-mediated truncation of vasoactive intestinal peptide (VIP), pituitary adenylyl cyclase-activating peptide (PACAP27 and PACAP38), gastrin-releasing peptide (GRP) and neuropeptide Y (NPY). DPPIV/CD26 sequentially cleaves off two dipeptides of VIP, PACAP27, PACAP38 and GRP. GRP situates between the best DPPIV/CD26 substrates reported, comparable to NPY. Surprisingly, the C-terminal extension of PACAP38, distant from the scissile bond, improves both PACAP38 binding and turnover. Therefore, residues remote from the scissile bond can modulate DPPIV/CD26 substrate selectivity as well as residues flanking it.

Key words: Dipeptidyl-peptidase IV; CD26; Gastrin-releasing peptide; Neuropeptide Y; Vasoactive intestinal peptide; Pituitary adenylyl cyclase-activating peptide

1. Introduction

The exopeptidase dipeptidyl-peptidase IV/CD26 (DPPIV/CD26; EC 3.4.14.5) [1] liberates X-Pro and X-Ala dipeptides from the amino-terminus of peptides. In addition, cleavage after a penultimate Ser, Gly, Thr, Val and Leu has been observed [2–5]. DPPIV/CD26 occurs in a soluble form in human serum and is expressed on the surface of endothelial and epithelial cells.

DPPIV/CD26 is involved in the regulation of specific peptides that serve as reporters within and between the digestive and endocrine system [6]. The enzyme rapidly inactivates glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP), both potent stimulators of glucose-induced insulin release [7]. The truncation of glucagon by DPPIV/CD26 has been demonstrated in vitro and the benefit of degradation-resistant analogs has been shown in a glucose tolerance bioassay [4,8]. Administration of DPPIV/CD26 inhibitors improves glucose tolerance in normal and diabetic animals [9–11]. DPPIV/CD26 inhibition in GLP-1 receptor −/− mice still increases significantly the glucose clearance rate [12]. This finding urged us to look for additional DPPIV/CD26 substrates involved in glucose homeostasis. DPPIV/CD26 is present in the granules of the glucagon-producing α-cells of the endocrine pancreas [13]. It is released together with glucagon in the interstitial space, where it can interact with secretory peptides of neighboring cells.

Parasympathetic nerve terminals in pancreatic ganglia and islets express the neuropeptides vasoactive intestinal peptide (VIP), pituitary adenylyl cyclase-activating peptide (PACAP) and gastrin-releasing peptide (GRP). They are released upon stimulation of the parasympathetic nerves and increase insulin secretion from the β-cells [14]. VIP, a 28-amino-acid peptide, and PACAP38, a 38-residue peptide with a C-terminally truncated 27-amino-acid variant (PACAP27), belong to the PACAP/glucagon superfamily [15]. With a penultimate serine and structural similarity to glucagon, VIP, PACAP27 and PACAP38 are potential DPPIV/CD26 substrates. Since the amino-terminal His is important for receptor binding, cleavage by DPPIV/CD26 would affect the biological activity [16,17].

GRP is a 27-amino-acid neuropeptide and member of the bombesin family of peptide hormones [18]. It stimulates the secretion of various gastrointestinal hormones [18–20] and is a regulator of the endocrine and exocrine pancreas [21,22]. Nausch et al. reported the sequential cleavage of two dipeptides of GRP by DPPIV/CD26, but no kinetic data were provided [23].

Neuropeptide Y (NPY), an acclaimed DPPIV/CD26 substrate [24], is widely distributed in the nervous system. This sympathetic cotransmitter is, aside from its cardiovascular effects, involved in the regulation of insulin release. DPPIV/CD26-truncation abolishes the binding of NPY with the pancreatic receptor and its vasoconstrictive properties, turning it into a vascular growth factor and feeding promoter [14,25].

We characterized the DPPIV/CD26-mediated truncation of VIP, PACAP27, PACAP38 and GRP. The truncation of NPY was used as a reference.

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Abbreviations: DPP, dipeptidyl-peptidase; GIP, glucose-dependent insulinotropic peptide; GLP, glucagon-like peptide; GRP, gastrin-releasing peptide; NPY, neuropeptide Y; PACAP, pituitary adenylyl cyclase-activating peptide; VIP, vasoactive intestinal peptide

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2. Materials and methods

2.1. Peptides

VIP and VIP(3–28) were synthesized using a solid-phase synthesizer model 433A (Applied Biosystems, Foster City, CA, USA). PACAP27, PACAP38 and GRP were purchased from Bachem (Bubendorf, Switzerland). GRP(3–27) was custom synthesized by Neosystem Labs (Strasbourg, France), NPY was purchased from Sigma (St. Louis, MO, USA). The concentration of the peptide stock solutions was determined spectrophotometrically using ε_{276} of 2900 M^{-1} cm^{-1} (VIP), 4350 M^{-1} cm^{-1} (PACAP27), 5800 M^{-1} cm^{-1} (PACAP38), 6850 M^{-1} cm^{-1} (GRP) and 7250 M^{-1} cm^{-1} (NPY).

2.2. DPPIV/CD26 purification and enzyme activity

Soluble DPPIV/CD26 was purified from human seminal plasma as described [26]. The specific activity was 35 U/mg. Contaminating aminopeptidase activity (measured with Ala-4-methoxy-2-naphthylamide) was $<0.1\%$. Enzymatic activity was determined kinetically at 37°C using Gly-Pro-*p*-nitroanilide (0.5 mM) in 40 mM Tris–HCl buffer, pH 8.3, at 405 nm (Spectramax 340 microtiterplate reader, Molecular Devices, Sunnyvale, CA, USA). One unit of activity is defined as the amount of enzyme that cleaves 1 μmole of substrate per minute under these conditions. Protein concentrations were determined using Bradford reagent with bovine serum albumin as a standard. The theoretical M_r of soluble DPPIV/CD26 is 85 123 per subunit.

2.3. Kinetics of peptide conversion

VIP, PACAP27, PACAP38, GRP and NPY (5 μM) were incubated with DPPIV/CD26 (25, 250 and 2500 U/l for VIP, PACAP27 and PACAP38; 0.25, 2.5 and 25 U/l for GRP and NPY) in 50 mM Tris–HCl buffer, pH 7.5, 1 mM EDTA at 37°C. At certain time intervals, samples were withdrawn and quenched in 0.1% trifluoroacetic acid. Incubation with buffer alone and with equal amounts of DPPIV/CD26 inactivated by diaryl phosphonate inhibitor, served as negative controls. The samples were desalted using C18 ZipTips (Millipore, Bedford, MA, USA) and eluted with 50 or 70% acetonitrile/0.1% acetic acid in water. The composition of the mixture was determined with an Esquire LC Ion Trap mass spectrometer (Bruker, Bremen, Germany) as described [5].

To determine the steady-state kinetic parameters, different concentrations of peptide (5–250 μM for VIP, 5–120 μM for PACAP27 and PACAP38, 5–100 μM for GRP and 0.625–80 μM for NPY) were incubated with DPPIV/CD26 for 5–30 min (10–25% conversion). The amount of converted substrate was calculated from the relative abundance of intact and cleaved forms. The average rate of conversion was plotted versus the average substrate concentration of the chosen time interval according to Segel [27]. The results were directly fitted to the Michaelis–Menten equation.

2.4. Competition experiments with chromogenic substrate

When two substrates (S and S′) are converted by the same enzyme (with normal Michaelis–Menten kinetics), the effect of the presence of S′ on the kinetics of S is:

$$v_i = \frac{V_{max}[S]}{K_m\left(1+\frac{[S']}{K_{m'}}\right)+[S]}$$

with V_{max} and K_m the maximal rate and Michaelis constant for S and K_m' the Michaelis constant for S′. As substrate S we used Gly-Pro-pNA (25 μM, $K_m = 100$ μM). K_m' was calculated from the initial rate in the absence and presence of a known concentration of peptide (S′).

3. Results

3.1. Specificity of cleavage, interpretation of mass spectra

The specificity of the reaction between DPPIV/CD26 and the peptides was confirmed by determining the composition of the reaction mixture in function of time. The observed molecular mass of intact and truncated forms is shown in Table 1. Cleavage occurred after the penultimate Pro for NPY and GRP and after the penultimate Ser for VIP, PACAP27 and PACAP38. VIP, PACAP27, PACAP38 and GRP were cleaved a second time, respectively, after Ala, Gly and Pro on position 4. Truncation was prevented by pre-incubation of DPPIV/CD26 with a specific irreversible inhibitor. The mass spectra only provide a relative abundance of the molecules in the sample. In order to quantify the amount of peptide converted at various time points, it was necessary to establish that the signal was proportional to the concentration and that this proportionality factor was the same for the intact and truncated molecules. Equal volumes of peptide dilutions, that had been incubated with either an excess of DPPIV/CD26 or the incubation buffer, were mixed, desalted and the ratio of the truncated over intact form was determined. In practice, the observed ratio approached the calculated ratio.

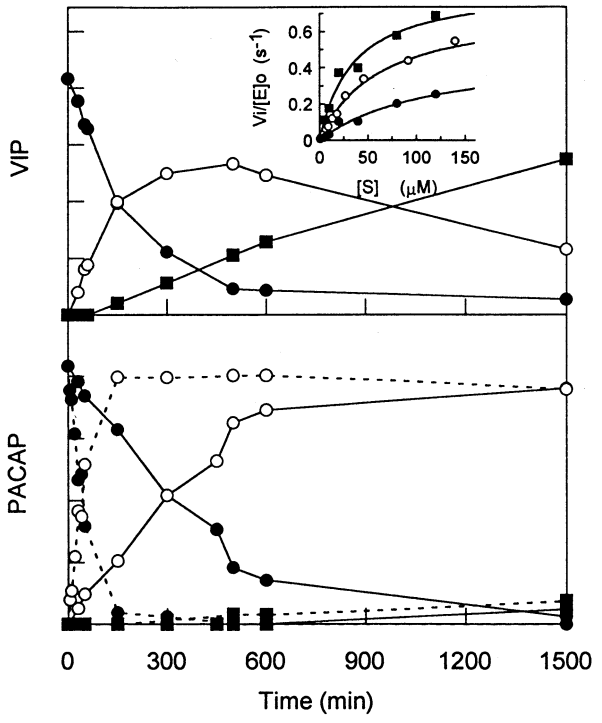

Fig. 1. Time course of degradation of neuropeptides (5 μM) by DPPIV/CD26 (25 U/l). Upper panel: VIP; lower panel: PACAP27 (solid lines) and PACAP28 (dotted lines) with intact peptide (closed circles), peptide-2 (open circles) and peptide-4 (closed squares). The inset shows the steady-state kinetics of VIP (open circles), PACAP27 (closed circles) and PACAP38 (closed squares) truncation by DPPIV/CD26.

3.2. Kinetics of peptide conversion

The time course of degradation of VIP, PACAP27, PACAP38, GRP and NPY is shown in Figs. 1 and 2. The C-terminal extension of PACAP38 accelerated the N-terminal truncation compared to PACAP27. The second truncation of VIP was, unexpected for cleavage after Ala, slower than the first since a large proportion of VIP(3–27) transiently accumulated. The removal of Asp-Gly of PACAP38(3–38) and PACAP27(3–27), was another 10–30 times slower than the cleavage after the VIP(3–27) N-terminal Asp-Ala. GRP was rapidly truncated by DPPIV/CD26 in two consecutive steps with little accumulation of the intermediate GRP(3–27) (Fig. 2).

The processing of NPY was also fast. We did not obtain the high kinetic constants reported before [28]. Our kinetic con-

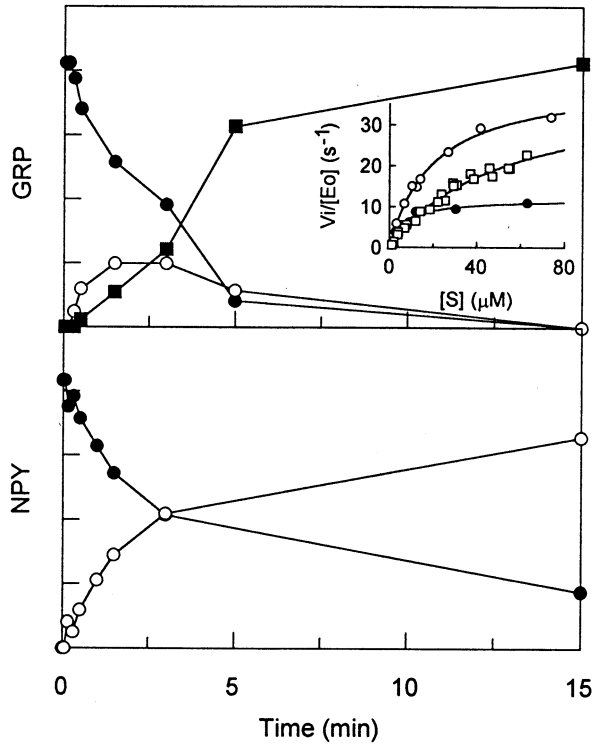

Fig. 2. Time course of degradation of neuropeptides (5 μM) by DPPIV/CD26 (25 U/l). Upper panel: GRP; lower panel: NPY with intact peptide (closed circles), peptide-2 (open circles) and peptide-4 (closed squares). The inset shows the steady-state kinetics of NPY (open squares), GRP (closed circles) and GRP(3–27) (open circles) truncation by DPPIV/CD26.

stants are approximately four-fold lower due to differences in (1) specific activity of the enzyme preparations used and (2) the molecular mass used in the calculations [26,29]. The quantification of peptide degradation requires calibration, adding another factor of uncertainty. The truncation of NPY did not deviate from Michaelis–Menten kinetics (Fig. 2, inset).

The catalytic rate constant (k_{cat}) and the K_m (Table 2) were determined from the concentration dependence of the conversion rate (Figs. 1 and 2). The K_m values derived from competition experiments with the chromogenic substrate support the mass spectrometric results.

Table 1
Specificity of peptide truncation by DPPIV/CD26

	N-terminus	Calculated M_r [a]	Measured M_r [b]
VIP	HSDAVF-	3325.8	3323.4 ± 1.7
	DAVF-	3101.6	3098.4 ± 1.0
	VF-	2915.4	2913.0 ± 1.3
PACAP27	HSDGIF-	3147.7	3147.4 ± 0.2
	DGIF-	2923.4	2923.3 ± 0.5
	IF-	2751.2	2751.3 ± 0.7
PACAP38	HSDGIF-	4534.2	4534.5 ± 0.2
	DGIF-	4310.0	4310.3 ± 0.6
	IF-	4137.9	4137.6 ± 0.8
GRP	VPLPAG-	2859.4	2857.3 ± 1.0
	LPAG-	2663.1	2661.2 ± 1.0
	AG-	2452.8	2451.1 ± 0.9
NPY	YPSKPD-	4271.7	4271.9 ± 0.4
	SKPD-	4011.4	4011.5 ± 0.6

[a]Theoretical average relative molecular mass of the peptides.
[b]Measured average relative molecular mass, the error represents the standard deviation of at least 20 measurements.

4. Discussion

DPPIV/CD26 is involved in the processing of several peptides belonging to the PACAP/glucagon family: glucagon [4,8], GLP-1 [7], GLP-2 [30], growth hormone-releasing factor (GRF) [2,3], peptide histidine methionine (PHM) and GIP [7]. The in vivo inactivation of GIP, GLP-1 and GLP-2 by DPPIV/CD26 has been demonstrated [31–33].

In vivo, bioactive peptides act in the pico- or nanomolar range [24]. At this low substrate concentration the most important parameter is the selectivity constant, k_{cat}/K_m. For the first truncation of VIP, we obtained a K_m value similar to the K_m of 56 μM published for [S^2,A^{15}]GRF(1–29)NH$_2$ [2] and a k_{cat} identical to the k_{cat} reported for glucagon [8]. Previously, it has been concluded that DPPIV is unable to cleave VIP in experimental conditions wherein GLP-1, PHM and GIP are readily truncated [7]. This is consistent with our results indicating a significantly lower k_{cat}/K_m value for VIP than for these substrates.

PACAP38 is identical to PACAP27, except for the strongly positively charged C-terminal extension. When the His[1] is removed, PACAP38(2–38) shows improved receptor binding and signalling compared to PACAP27(2–27) [16]. Surprisingly, PACAP38 has a higher binding affinity for DPPIV/CD26 and a higher turnover rate. Thus the C-terminal extension contributes to the interaction with DPPIV/CD26 and influences the active site conformation during the rate-determining catalytic step. Therefore, it is difficult to predict the substrate specificity of a peptide exclusively from the amino acid sequence surrounding the scissile bond [5]. The study of the processing of GRF-analogs also suggested that DPPIV/CD26 interacts with substrate residues remote from the scissile bond [2].

The k_{cat}/K_m of GRP is significantly higher than for GLP-1 and GIP, whose active intact peptide levels are regulated by DPPIV/CD26 in vivo. The truncated GRP(5–27) form has been isolated from dog intestine [34]. It is possible that GRP, after sequential cleavage by DPPIV/CD26, is rapidly degraded by other peptidases to shorter forms [35–37]. The

Table 2
Steady-state kinetics for peptide truncation by DPPIV/CD26

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
VIP	59 ± 14	0.74 ± 0.06	12 000 ± 2000
VIP(3–28)	ND	ND	1700 ± 50[c]
PACAP27	120 ± 50	0.49 ± 0.13	4200 ± 800
PACAP27 (3–27)	ND	ND	100 ± 50[c]
PACAP38	35 ± 9	0.85 ± 0.09	24 000 ± 4000
PACAP38 (3–38)	ND	ND	150 ± 75[c]
GRP	6 ± 1	11 ± 1	$1.8\times10^6 \pm 3\times10^5$
	14 ± 11[a,b]		
GRP(3–27)	20 ± 2	41 ± 2	$2.1\times10^6 \pm 1\times10^5$
	20 ± 1[a]		
NPY	52 ± 8	40 ± 3	$7.6\times10^5 \pm 5\times10^4$
	37[a]		

The errors represent the standard error on the fit.
[a]K_m value determined from competition experiments.
[b]This value likely contains a contribution of GRP(3–27) since the second cleavage is faster than the first.
[c]Calculated from the time course of product formation at a single substrate concentration assumed to be $\ll K_m$.

penultimate Pro of GRP is conserved in evolution but no function has been attributed to it. During future in vivo studies of GRP it might be interesting to compare its functional activities in the presence and absence of DPPIV activity (DPPIV/CD26-deficient animals or in vivo inhibition).

It is clear that this in vitro kinetic study primarily contributes to the understanding of the DPPIV/CD26 enzymatic action and substrate selectivity. Obviously the in vivo half-life of a peptide depends on many factors, including cleavage by different exo- and endo-peptidases, cellular uptake, renal clearance and hepatic metabolism. VIP is rapidly cleared from plasma in men. A significant part of the clearance is attributable to the liver [38,39]. The local concentration of DPPIV/CD26 and competition between different substrates may greatly influence the turnover of a given peptide.

In conclusion, DPPIV/CD26 truncates VIP, PACAP27 and PACAP38 in two consecutive steps. The kinetic constants are comparable to those reported for homologous peptides. Furthermore, we found that GRP and GRP(3–27) are excellent DPPIV/CD26 substrates. The finding that the positive C-terminal extension of PACAP38, remote from the scissile bond, can influence both substrate binding and the catalytic rate constant has important consequences for the concept of DPPIV/CD26 substrate specificity and catalysis.

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